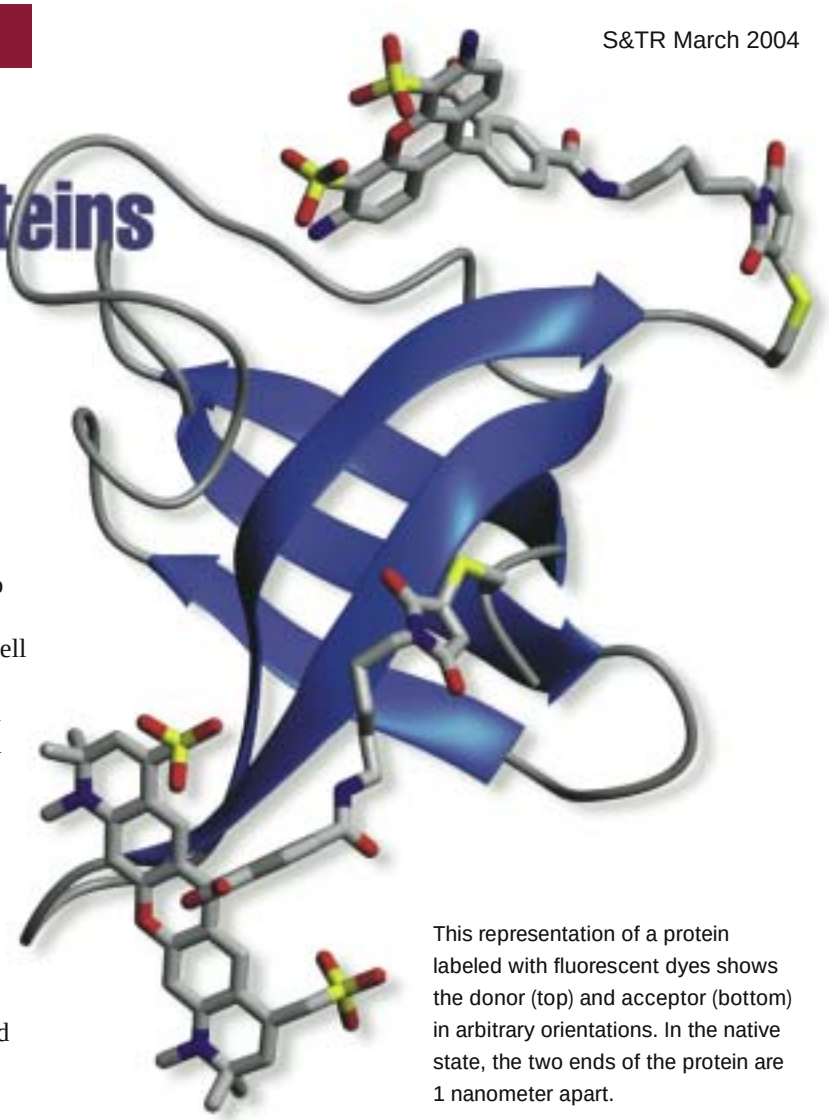
Observing How Proteins Loop the Loop



This representation of a protein labeled with fluorescent dyes shows the donor (top) and acceptor (bottom) in arbitrary orientations. In the native state, the two ends of the protein are 1 nanometer apart.

THE stretched-out lazy chains of amino acids forming the proteins that make life will, with the right chemical prodding, suddenly twist and tie into loopy, intricate three-dimensional shapes, like a complicated shoelace. And not just any shape will do: A backward twist or a wrong knot can cause something to go horribly wrong. A misfolded protein can poison the surrounding cells, leading to diseases such as Alzheimer's and mad cow as well as some cancers.

To better understand this process, Livermore physicist Olgica Bakajin worked with scientists from the Laboratory of Chemical Physics at the National Institute of Diabetes and Digestive and Kidney Diseases, which is part of the National Institutes of Health, and the Physikalische Biochemie at Universität Potsdam in Germany, to develop a method for investigating protein folding one molecule at a time. Their observations are the first of protein-folding kinetics on the single-molecule level. Using a microfluidic mixer they developed, the scientists were able to look at a protein at defined times after the folding reaction began and, for the first time, isolate a short-lived "collapsed state."

"The team is interested in how a protein goes from a random coil to its functional folded form," says Bakajin. "With our instrument, researchers will come closer to understanding this process and be able to study many different proteins to come up with some general rules about how proteins fold."

Detect the Subtlest Fold

Bakajin designed and built the mixer with support from Livermore's Laboratory Directed Research and Development Program. The device includes microchannels for introducing various solutions, a region for mixing protein and chemicals, and a chamber that allows researchers to record data. The microchannels, 8 to 50 micrometers deep and 5 to 50 micrometers wide, are cut into the surface of a silicon wafer. A solution consisting of protein and denaturant, which keeps the protein structure "relaxed" and unfolded, is fed into the center channel. A buffer is then fed into the two outer channels, to dilute the denaturant and allow the protein to fold. Compressed air drives the solutions through the channels at a specified flow rate. When the solutions contact each other, they mix, dilute the denaturant, and within a few tens of milliseconds, initiate the protein-folding reaction.

To demonstrate the performance of the mixer, the researchers used a "cold shock" protein from the bacterium *Thermotoga maritime*, a thermophile organism that lives in some hot springs. This particular protein, notes Bakajin, has two recognized stable states: folded and unfolded. For this experiment, the team labeled each end of the protein with fluorescent dye. When the protein is excited by 488-nanometer laser light, one dye molecule, called the donor, emits light at a specific wavelength. The other dye molecule, called the acceptor, absorbs the donor light and then emits light of a different wavelength.

Bakajin explains, "If the two dye molecules are far apart—such as when the protein is in its relaxed, unfolded state—not much donor energy reaches the acceptor. If the two molecules are close—that is, when the protein is folded—more of the donor's energy reaches the acceptor, and the intensity of the acceptor's emitted light increases. So the ratio of emission between these two molecules tells us how far apart the dye molecules are and lets us know the state of the protein."

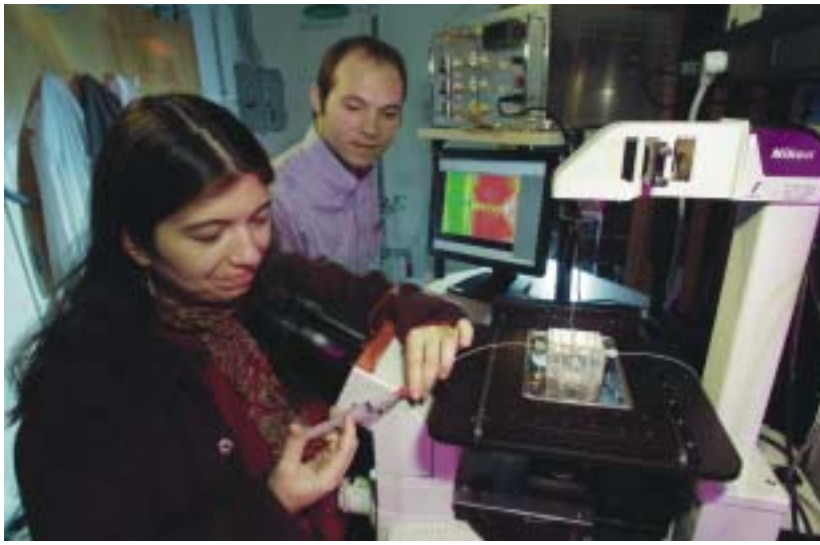
In the mixing region, once the denaturant is diluted, the protein begins to fold. The 488-nanometer laser beam is positioned downstream to illuminate a section of the observation channel that has been marked at specific intervals. Because the protein mixture

flows down the tube at a known velocity, each mark corresponds to a particular time delay after mixing. Photon detectors aimed at these marked areas collect data in millisecond intervals as individual molecules pass by. In this way, the team was able to collect data on the status of individual protein molecules and determine whether a molecule was folded or unfolded. "Before now," says Bakajin, "no one has been able to look at individual proteins under nonequilibrium conditions in this manner."

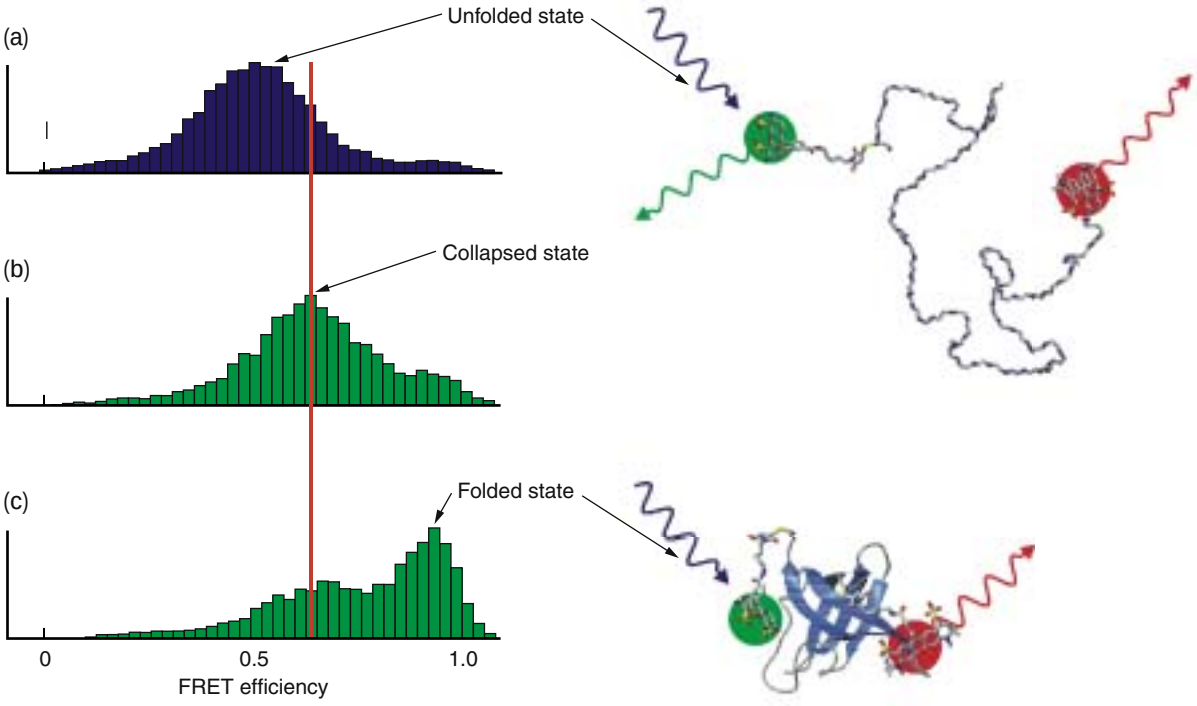
From these data, the team identified a short-lived, interim state between the unfolded and folded state. The interim state is apparent at 100 milliseconds—the time of the team's first observation. In this state, the molecule has become more compact or collapsed, but it is still in a random coil and not in its functional folded state. Bakajin notes that no one has directly observed and isolated this collapsed state before. "In principle, we should be able to study the properties of this very short lived state because it is separated from the folded state in our experiments."

The signals for each state—the initial unfolded, uncollapsed state; the interim collapsed state; and the final folded state—are quite different. Each is characterized by a different transfer efficiency—the rate that energy is transferred from the donor to the acceptor. The processes of collapse and folding can also be distinguished from each other. As shown in the figure below, the peak shifts from the lower transfer efficiency that corresponds to the unfolded, uncollapsed state to a higher efficiency, which corresponds to the collapsed state. During folding, the population of the collapsed state decreases while the population of the folded state increases. These changes alter the peak heights, which correspond to the protein-folding states, but the position of the peaks remain the same.

The microfluidic device that Bakajin and her colleagues developed allows scientists to monitor protein-folding kinetics in millisecond snapshots. But to clarify some of the faster events, scientists want to take measurements on time scales



Olgica Bakajin (left) and David Hertzog characterize the microfluidic mixers at Livermore's Biosecurity and Nanosciences Laboratory.



Histograms of the Forster Resonance Energy Transfer (FRET) efficiency, E_m, which is used to measure protein folding. As the fluorescent donor and emitter molecules move closer to each other, E_m increases, and higher E_m means more folded proteins. The red line shows the mean value for E_m in the unfolded state after mixing. (a) Protein is in equilibrium at the start of the experiment—most of the observed molecules are unfolded. (b) At 100 milliseconds, the peak has shifted, showing that the ends are closer together, but they are not folded. This is the interim, or collapsed, state. (c) By 1 second, E_m indicates that most of the proteins are in the folded state.

that are 1,000 times shorter. Present technology does not allow observation of single molecules at these extremely short time scales because the single dye molecules simply don't emit photons fast enough. Such measurements can be done by looking at many molecules at the same time, but for scientists to observe events on the microsecond scale, the protein-folding reaction must be initiated within that time frame.

To solve this problem, David Hertzog, a mechanical engineering graduate student at Stanford University, is working with Bakajin on a new microfluidics device that mixes reagents and initiates protein-folding reactions in microseconds. In this device, fluids travel as fast as 1 meter per second through a 10-micrometer-wide channel. Because of the tiny dimensions, miniscule amounts of protein are sufficient to perform measurements: 10 microliters is enough to run experiments continuously for 3 hours. Researchers are also working to improve the mixing time and background levels of the mixers for single molecule observations. Instead of using a tiny observation window of glass—which fluoresces and introduces some background photons—they are experimenting with windows of fused silica.

Predicting with Precision

To get a better handle on how the various parts of the string fold, says Bakajin, scientists plan to insert the donor and acceptor dye molecules in different areas of the protein strings. Another experiment researchers want to perform is to introduce mutations into the proteins or cut off parts of the chain and then see how those changes affect the folding process. The hope is that, given a sequence of amino acids and a specific environment, researchers will be able to predict not only a protein's final shape but also how it got there.

"This is important information for understanding and eventually treating diseases that result from misfolded proteins and for such ventures as creating designer drugs," says Bakajin. "Researchers believe that the folding process sometimes becomes derailed in the collapsed or interim states due to increased temperatures or acidity or other such factors. The Laboratory will be part of bringing this understanding to light through its novel microtechnology."

She points out that this project is also an example of how Livermore researchers collaborate with scientists and organizations outside the Laboratory, to develop technology that will also be useful for other Livermore projects. "Microfluidics is crucial for developing miniature detectors and sensors of all kinds," says Bakajin. "Such devices will be used not only in basic science research but also for homeland security applications. This project is just one of the opportunities we have to contribute to basic science using microfluidic technologies developed at Livermore."

—Ann Parker

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